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*Published in:*  
Applied and environmental microbiology

*DOI:*  
[10.1128/AEM.02254-12](https://doi.org/10.1128/AEM.02254-12)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2013

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### *Citation for published version (APA):*

Pudlik, A. M., & Lolkema, J. S. (2013). Uptake of alpha-Ketoglutarate by Citrate Transporter CitP Drives Transamination in *Lactococcus lactis*. *Applied and environmental microbiology*, 79(4), 1095-1101.  
<https://doi.org/10.1128/AEM.02254-12>

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# Uptake of $\alpha$ -Ketoglutarate by Citrate Transporter CitP Drives Transamination in *Lactococcus lactis*

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**Transamination is the first step in the conversion of amino acids into aroma compounds by lactic acid bacteria (LAB) used in food fermentations. The process is limited by the availability of  $\alpha$ -ketoglutarate, which is the best  $\alpha$ -keto donor for transaminases in LAB. Here, uptake of  $\alpha$ -ketoglutarate by the citrate transporter CitP is reported. Cells of *Lactococcus lactis* IL1403 expressing CitP showed significant levels of transamination activity in the presence of  $\alpha$ -ketoglutarate and one of the amino acids Ile, Leu, Val, Phe, or Met, while the same cells lacking CitP showed transamination activity only after permeabilization of the cell membrane. Moreover, the transamination activity of the cells followed the levels of CitP in a controlled expression system. The involvement of CitP in the uptake of the  $\alpha$ -keto donor was further demonstrated by the increased consumption rate in the presence of L-lactate, which drives CitP in the fast exchange mode of transport. Transamination is the only active pathway for the conversion of  $\alpha$ -ketoglutarate in IL1403; a stoichiometric conversion to glutamate and the corresponding  $\alpha$ -keto acid from the amino acids was observed. The transamination activity by both the cells and the cytoplasmic fraction showed a remarkably flat pH profile over the range from pH 5 to pH 8, especially with the branched-chain amino acids. Further metabolism of the produced  $\alpha$ -keto acids into  $\alpha$ -hydroxy acids and other flavor compounds required the coupling of transamination to glycolysis. The results suggest a much broader role of the citrate transporter CitP in LAB than citrate uptake in the citrate fermentation pathway alone.**

Transamination is the central reaction in amino acid catabolic pathways in lactic acid bacteria (LAB) that results in the formation of aroma compounds in food fermented products. The reaction is catalyzed by aminotransferases and results in the formation of the corresponding  $\alpha$ -keto acids from amino acids, while an  $\alpha$ -keto donor (e.g.,  $\alpha$ -ketoglutarate) is transformed to the corresponding amino acid (i.e., glutamate [Glu]). Subsequently,  $\alpha$ -keto acids are degraded to flavor compounds like aldehydes, alcohols, carboxylic acids, or  $\alpha$ -hydroxy acids by additional (enzymatic or chemical) reactions.  $\alpha$ -Ketoglutarate is reported to be the best keto donor for transamination by the common dairy starter bacterium *Lactococcus lactis* (1, 2). Two major  $\alpha$ -ketoglutarate transaminases involved in flavor development in *L. lactis* have been identified and characterized. BcaT converts branched-chain amino acids and methionine (1); AraT converts aromatic amino acids, methionine, and leucine (3). A double knockout of the two genes encoding these transaminases, *bcaT* and *araT*, respectively, showed that another transaminase is involved in the conversion of aspartate (4). The latter is transaminated by aspartate transaminase AspC, encoded by the *aspB* gene in *L. lactis* IL1403 (5, 6). All three enzymes belong to the same family of pyridoxal 5'-phosphate (PLP)-dependent transaminases.

The conversion of amino acids into aroma compounds by LAB in cheese is often limited by the lack of  $\alpha$ -ketoglutarate. Only a few strains of *L. lactis* are capable of producing  $\alpha$ -ketoglutarate by deamination of glutamate, which is present in large quantities in milk, catalyzed by glutamate dehydrogenase (7). The other alternative to enhance cheese aroma would be addition of external  $\alpha$ -ketoglutarate (4). Unfortunately, external  $\alpha$ -ketoglutarate must be provided at a high concentration to result in enhanced flavor compound production, possibly because of the lack of a system for uptake of  $\alpha$ -ketoglutarate.

CitP, the citrate uptake system in LAB, was previously characterized in *L. lactis* (8–10) and *Leuconostoc mesenteroides* (11, 12).

The primary function of CitP is uptake of citrate during citrate/carbohydrate cometabolism when CitP catalyzes the uptake of citrate into the cell in exchange with the end product of glycolysis, L-lactate (precursor/product exchange) (11, 12). Studies in resting cells showed that under conditions when L-lactate was not present, citrate was taken up by CitP in exchange with the citrate metabolic pathway intermediates/end products pyruvate,  $\alpha$ -acetolactate, and/or acetate (9). Similar studies demonstrated the affinity of CitP for oxaloacetate, another intermediate of citrate metabolism, by identifying the role of CitP in oxaloacetate metabolism (10) and citrate-driven transamination in an oxaloacetate decarboxylase-deficient mutant of *L. lactis* IL1403 (13). The different metabolic functions of CitP were consistent with previous citrate transport studies *in vitro* using right-side-out (RSO) membrane vesicles derived from *L. lactis*, in which the transporter was demonstrated to translocate 2-hydroxycarboxylates (citrate, L-lactate,  $\alpha$ -acetolactate) and 2-ketocarboxylates (pyruvate and oxaloacetate) (11) but also showed a new substrate, the carboxylate acetate (9). Subsequently, a systematic study of the substrate specificity of the transporter characterized CitP as a very promiscuous carboxylate transporter that translocates mono-, di-, and tricarboxylates of the form  $X-CR_2-COO^-$ , in which X is either OH, O, or H but not  $NH_3^+$  (amino acids), and with a preference for  $OH > O > H$  at the C-2 atom (15). Many of the monocarboxylate substrates are flavor compounds or precursors thereof derived from amino acid catabolic pathways, i.e.,  $\alpha$ -keto acids and  $\alpha$ -hydroxy acids. In ad-

Received 27 August 2012 Accepted 28 November 2012

Published ahead of print 30 November 2012

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doi:10.1128/AEM.02254-12

dition, many metabolites like the hydroxy (OH), keto (O), and acid (H) forms of  $C_4$  dicarboxylates (i.e., L-malate, oxaloacetate, succinate) and  $C_5$  and  $C_6$  dicarboxylates (glutarate and adipate, respectively) are transported by CitP (11, 15).

In the present study, it was first demonstrated that  $\alpha$ -ketoglutarate-driven transamination by *L. lactis* IL1403 is limited by the lack of an uptake system for  $\alpha$ -ketoglutarate. Subsequently, it is shown that the limitation is overcome in *L. lactis* IL1403(pFL3), a recombinant strain that expresses the citrate transporter CitP. CitP is shown to recognize and catalyze the uptake of  $\alpha$ -ketoglutarate into the cell. The study emphasizes the potentially broad role of the citrate transporter CitP in flavor compound production by *Lactococcus lactis* subsp. *lactis* biovar diacetylactis.

## MATERIALS AND METHODS

**Chemicals.** 2-Hydroxy-4-methylthiobutyrate, 2-keto-3-methylvalerate, 2-keto-4-methylthiobutyrate, aldehyde dehydrogenase, diethylethoxymethylenemalonate (DEEMM), L-alanine, L-aminoadipic acid, L-cysteine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, methyl-3-methylthiopropionate, nisin, phenyllactate, phenylpyruvate, PLP,  $\alpha$ -ketoglutarate,  $\alpha$ -ketoisocaproate, and  $\alpha$ -ketoisovalerate were obtained from Sigma-Aldrich Chemicals. L-Arginine, L-asparagine, L-aspartate, L-glutamate, L-glycine, and L-methionine were obtained from Merck. Solutions were prepared in 50 mM potassium phosphate (pH 5.8) buffer; if required, the pH was adjusted to 5.8 with 5 M KOH.

**Bacterial strains and growth conditions.** Strains *Lactococcus lactis* IL1403 (16), IL1403(pFL3) (8), and IL-9000(pNZ-CitP) (17) were used in this study. Plasmid pFL3 harbors the lactococcal *citP* gene under the control of the *Streptococcus pneumoniae* *polA* promoter (8). The *citP* gene in pFL3 originates from the natural strain of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis CRL264 isolated from cheese (18). Neither expression nor plasmid copy number is under the control of citrate or pH in strain IL1403(pFL3) (19). *L. lactis* IL-9000 is an IL1403 derivative in which the NICE (nisin-controlled expression) system for nisin-inducible gene expression (*nisRK*) is employed (17). The *citP* gene in plasmid pNZ8048 is under the control of the nisin-inducible promoter *nisA* (20). Precultures were grown overnight at 30°C in M17 broth medium supplemented with 0.5% (wt/vol) glucose (M17G) and 5  $\mu\text{g} \cdot \text{ml}^{-1}$  of tetracycline or chloramphenicol, if required. Cells were grown in M17G with the initial pH adjusted to 7.0. If required, nisin was added at different concentrations ranging from 0 to 10 ng  $\cdot \text{ml}^{-1}$ . Growth was performed in 100-ml serum bottles without agitation and at 30°C. Growth was followed by measuring the optical density at a wavelength of 660 nm ( $\text{OD}_{660}$ ). Cells were harvested at mid-exponential growth phase when the optical density was 0.6 by centrifugation for 10 min at 2,000 relative centrifugal force ( $2,000 \times g$ ). Cells were washed two times with 50 mM potassium phosphate (pH 5.8) buffer, centrifuged at 4°C, and finally, resuspended in the same buffer at 4°C.

**$\alpha$ -Ketoglutarate-driven transamination.** Resting cells at an  $\text{OD}_{660}$  of 1.5 in 50 mM potassium phosphate (pH 5.8) buffer were incubated at 30°C without agitation for 10 min. The assay was performed in a total volume of 1.5 ml. At time zero, 2 mM  $\alpha$ -ketoglutarate was added in the presence or absence of 2 mM amino acid and 50  $\mu\text{M}$  PLP. Samples of 100  $\mu\text{l}$  were taken at the indicated times and immediately centrifuged for 0.5 min at maximum speed in a tabletop centrifuge. The supernatant was stored on ice or frozen until further analysis by high-pressure liquid chromatography (HPLC)/reverse-phase HPLC (RP-HPLC).

**HPLC/RP-HPLC analysis.** Samples were run on a Shimadzu high-speed HPLC Prominence ultrafast liquid chromatograph and later analyzed using LC Solutions (version 1.24) SP1 software from Shimadzu (Kyoto, Japan).  $\alpha$ -Ketoglutarate and products of  $\alpha$ -ketoglutarate-driven transamination ( $\alpha$ -keto acids and  $\alpha$ -hydroxy acids) were determined by loading an aliquot of 10  $\mu\text{l}$  of the supernatant on an Aminex HPX-87H anion-exchange column with dimensions of 300 by 7.8 mm (Bio-Rad

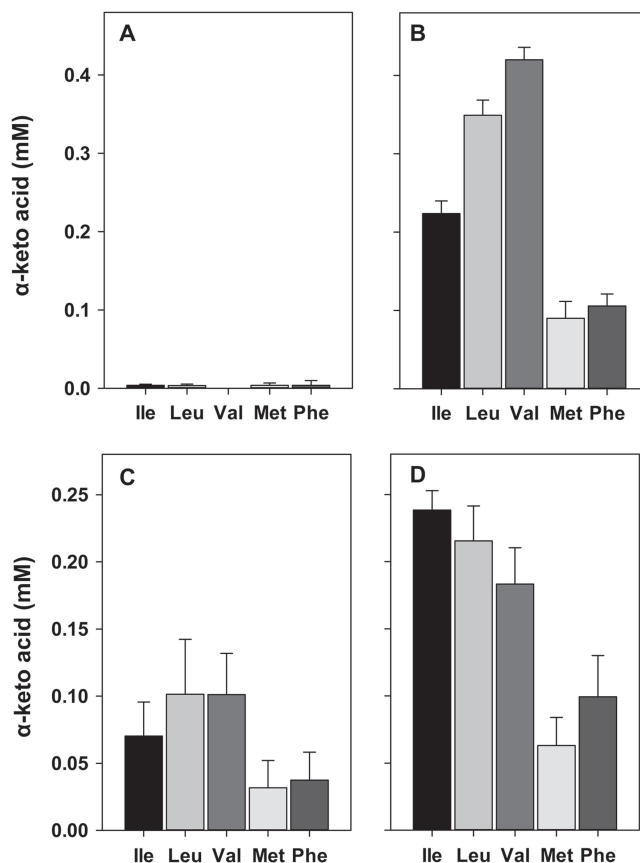


FIG 1  $\alpha$ -Ketoglutarate-driven transamination by *L. lactis* strains IL1403 (A, B) and IL1403(pFL3) (C, D). (A, C) Transamination by resting cells; (B) transamination by permeabilized cells; (D) transamination in the presence of 0.2 mM L-lactate. Concentrations of the corresponding  $\alpha$ -keto acids from Ile, Leu, Val, Phe, and Met were determined after 3 h of incubation in the presence of 2 mM  $\alpha$ -ketoglutarate, 2 mM amino acids, and 50  $\mu\text{M}$  PLP.

Laboratories, Inc., Richmond, CA) operated at 30°C in isocratic mode using 0.005 M  $\text{H}_2\text{SO}_4$  as the mobile phase and a flow rate of 0.8 ml/min.

Amino acids were analyzed by RP-HPLC after DEEMM derivatization as described before (13). Briefly, aminoenone derivatives of amino acids were obtained by reaction of the supernatant with 1 M borate (pH 9.0) buffer, methanol, and DEEMM in a closed tube over 30 min incubation at room temperature in an ultrasound bath. Then, the sample was incubated at 70°C to allow complete degradation of the excess of DEEMM. Detection of aminoenone derivatives was performed in an Alltech Platinum EPS  $C_{18}$  column with dimensions of 250 by 4.6 mm operated at 25°C through the binary gradient with a flow rate of 0.8 ml/min. Eluent A was 25 mM acetate (pH 5.8) buffer with 0.02% sodium azide, and eluent B was an 80:20 mixture of acetonitrile and methanol. The target compounds were identified according to the retention times and were quantified using the external standard method. Mean values and standard deviations were calculated from at least 3 different independent experiments.

## RESULTS

**Transamination of  $\alpha$ -ketoglutarate in *L. lactis* IL1403.** No  $\alpha$ -keto acids were formed by resting cells of *L. lactis* IL1403 from any of the amino acids Ile, Leu, Val, Phe, or Met after 3 h of incubation in the presence of  $\alpha$ -ketoglutarate (Fig. 1A). The result was the same when, in addition, 1 mM glucose was present to energize the cells (not shown). The experiment was repeated after

TABLE 1 Efficiency of  $\alpha$ -ketoglutarate-driven transamination by *L. lactis* IL1403(pFL3)

Strain	Incubation time (h)	Amino acid <sup>a</sup>	Amt ( $\mu$ M)		Efficiency <sup>d</sup> (%)
			$\alpha$ -kg <sup>b</sup>	$\alpha$ -ka <sup>c</sup>	
<i>L. lactis</i> IL1403(pFL3) (resting cells)	3	Ile	96 $\pm$ 6	92 $\pm$ 7	96 $\pm$ 13
	3	Leu	97 $\pm$ 8	96 $\pm$ 3	99 $\pm$ 11
	3	Val	95 $\pm$ 5	96 $\pm$ 6	99 $\pm$ 11
	3	Met	39 $\pm$ 5	39 $\pm$ 5	100 $\pm$ 25
	3	Phe	44 $\pm$ 5	44 $\pm$ 5	100 $\pm$ 23
	24	Ile	930 $\pm$ 93	985 $\pm$ 84	106 $\pm$ 19
	24	Leu	1,023 $\pm$ 101	1,011 $\pm$ 89	99 $\pm$ 19
	24	Val	1,159 $\pm$ 87	1,117 $\pm$ 88	96 $\pm$ 15
	24	Met	471 $\pm$ 82	457 $\pm$ 52	97 $\pm$ 28
	24	Phe	411 $\pm$ 52	417 $\pm$ 62	101 $\pm$ 28
<i>L. lactis</i> IL1403/IL1403(pFL3) <sup>e</sup> (permeabilized cells)	3	Ile	254 $\pm$ 27	224 $\pm$ 19	88 $\pm$ 18
	3	Leu	331 $\pm$ 30	353 $\pm$ 32	106 $\pm$ 19
	3	Val	442 $\pm$ 52	420 $\pm$ 29	95 $\pm$ 18
	3	Met	111 $\pm$ 12	101 $\pm$ 11	91 $\pm$ 21
	3	Phe	95 $\pm$ 11	110 $\pm$ 13	115 $\pm$ 25
	24	Ile	1,710 $\pm$ 68	1,802 $\pm$ 70	105 $\pm$ 8
	24	Leu	1,821 $\pm$ 107	1,826 $\pm$ 101	100 $\pm$ 11
	24	Val	1,920 $\pm$ 96	1,798 $\pm$ 98	94 $\pm$ 10
	24	Met	1,069 $\pm$ 57	1,066 $\pm$ 87	100 $\pm$ 13
	24	Phe	1,040 $\pm$ 67	1,100 $\pm$ 99	105 $\pm$ 16

<sup>a</sup> Amino acids were added at a concentration of 2 mM.<sup>b</sup> Amount of  $\alpha$ -ketoglutarate ( $\alpha$ -kg) consumed.<sup>c</sup> Amount of  $\alpha$ -keto acid ( $\alpha$ -ka) produced from the amino acid.<sup>d</sup> Percentage of  $\alpha$ -ketoglutarate converted to the  $\alpha$ -keto acid.<sup>e</sup> Similar activities were observed with the two strains; the data were pooled.

permeabilization of the cells, which omits all transport steps. With permeabilized cells, the corresponding  $\alpha$ -keto acids of Ile, Leu, Val, Met, and Phe were formed at concentrations of 230, 350, 420, 100, and 100  $\mu$ M, respectively (Fig. 1B). Almost complete conversion of  $\alpha$ -ketoglutarate was observed after 24 h of incubation in the presence of the branched-chain amino acids, whereas conversion of Met and Phe was about two times slower (Table 1). The amounts of  $\alpha$ -ketoglutarate consumed and  $\alpha$ -keto acids produced were in good agreement, indicating that other pathways for  $\alpha$ -ketoglutarate conversion were not active in permeabilized cells of *L. lactis* IL1403. Control experiments performed in the absence of  $\alpha$ -ketoglutarate did not result in the formation of the  $\alpha$ -keto acids, and omitting PLP from the incubation mixture strongly decreased the production of the  $\alpha$ -keto acids (not shown). Hence, PLP-dependent transaminases are active in the cytoplasm of *L. lactis* IL1403 with  $\alpha$ -ketoglutarate as the keto donor and the amino acids Ile, Leu, Val, Phe, and Met. Most importantly,  $\alpha$ -ketoglutarate-driven transamination by resting cells was limited by the uptake and/or excretion of one or more of the substrates and products.

**$\alpha$ -Ketoglutarate-driven transamination by *L. lactis* IL1403(pFL3).**  $\alpha$ -Ketoglutarate-driven transamination was assayed in resting cells of *L. lactis* IL1403(pFL3), which expresses the citrate transporter gene *citP* located on plasmid pFL3. Plasmid pFL3 contains the lactococcal *citP* gene under the control of the constitutive promoter *polA* from *S. pneumoniae* (8). Neither expression level nor plasmid copy number is affected by the presence of citrate or the pH of the growth medium (19), resulting in a constant level of expression of CitP. In contrast to strain IL1403, resting cells of IL1403(pFL3) produced significant amounts of  $\alpha$ -keto acids when

incubated with  $\alpha$ -ketoglutarate in the presence of each of the amino acids Ile, Leu, Val, Met, or Phe and PLP (Fig. 1C). After 3 h, 90 to 100  $\mu$ M  $\alpha$ -ketomethylvalerate,  $\alpha$ -ketoisocaproate, and  $\alpha$ -ketoisovalerate were produced in the presence of Ile, Leu, and Val, respectively, while in the presence of Met and Phe, the amounts were smaller: 40 to 45  $\mu$ M 2-keto-4-methylthiobutyrate and phenylpyruvate, respectively. The amounts increased 10-fold after 24 h of incubation (Table 1). Similarly, as observed with permeabilized cells of IL1403, the amounts of  $\alpha$ -ketoglutarate consumed and  $\alpha$ -keto acids produced at both time points were in good agreement, suggesting no other pathway for  $\alpha$ -ketoglutarate, except for transamination, in resting cells as well. No formation of  $\alpha$ -keto acids was observed without addition of a keto donor (not shown). The transamination activities of permeabilized cells of the recombinant and parental strains were not significantly different (Table 1).

Production of Glu by resting cells of the recombinant strain under conditions identical to those described above was measured by RP-HPLC. Cells incubated for 3 h without further additions, with 2 mM  $\alpha$ -ketoglutarate, or with 2 mM Ile produced 30 to 40  $\mu$ M Glu. In addition, low concentrations ( $\sim$ 10  $\mu$ M) of Asp, Asn, Gln, Gly, Thr, and Lys were detected, suggesting some proteolytic activity of the cells, as was observed before (data not shown) (13). In the presence of both  $\alpha$ -ketoglutarate and Ile, the amount of Glu produced by the cells was raised significantly to 120  $\mu$ M (Table 2). The same experiment was performed with the other 4 amino acids, Leu, Val, Phe, and Met, and in all cases, an increase in the production of Glu was observed when both  $\alpha$ -ketoglutarate and the amino acid were present (Table 2). The amounts of  $\alpha$ -ketoglutarate consumed and Glu and  $\alpha$ -keto acids produced were in



TABLE 2  $\alpha$ -Ketoglutarate-driven transamination in *L. lactis* IL1403(pFL3)

Amino acid <sup>a</sup>	Amt of Glu ( $\mu$ M) <sup>b</sup>
Ile	95 $\pm$ 5
Leu	95 $\pm$ 5
Val	98 $\pm$ 5
Phe	63 $\pm$ 5
Met	35 $\pm$ 8

<sup>a</sup> Amino acids were added at a concentration of 2 mM.  
<sup>b</sup> The amount of Glu was measured after 3 h and corrected for the amounts produced by proteolytic activity.

good agreement (Tables 1 and 2). Hence, resting cells of *L. lactis* IL1403 expressing the citrate transporter CitP revealed enhanced transamination activity driven by external  $\alpha$ -ketoglutarate.

Enhancement of the rate of uptake of a substrate by L-lactate is diagnostic for substrates of CitP (9, 10, 12). In the presence of 0.2 mM L-lactate, resting cells of *L. lactis* IL1403(pFL3) incubated with 2 mM  $\alpha$ -ketoglutarate and 2 mM the amino acids showed an increased production of the corresponding  $\alpha$ -keto acids by at least a factor of 2 after 3 h (Fig. 1D). The amounts of  $\alpha$ -keto acids formed by resting cells of IL1403(pFL3) in the presence of L-lactate and by the same cells permeabilized with Triton X-100 showed that in the presence of L-lactate, uptake of  $\alpha$ -ketoglutarate still limited the rate of the reaction (compare Table 1 and Fig. 1). Detailed kinetics of the production of  $\alpha$ -ketomethylvalerate from Ile in the presence and absence of L-lactate showed that the rate of formation was significantly higher from the beginning in its presence (4.0 and 0.5  $\mu$ M  $\cdot$  min<sup>-1</sup>, respectively) (Fig. 2). No  $\alpha$ -keto acids were formed when the cells were incubated with L-lactate and the amino acids in the absence of  $\alpha$ -ketoglutarate (not shown). Also, cells of *L. lactis* IL1403 not expressing CitP did not show formation of  $\alpha$ -ketomethylvalerate when incubated with  $\alpha$ -ketoglutarate, L-lactate, and the amino acids.

**Optimization of  $\alpha$ -ketoglutarate-driven transamination by controlled expression of CitP.** The transamination activity of strain IL-9000(pNZ-CitP), which contains the *citP* gene under the control of the nisin-inducible promoter *nisA* (17), was optimal when the cells were grown in the presence of 0.5 to 5 ng  $\cdot$  ml<sup>-1</sup> of

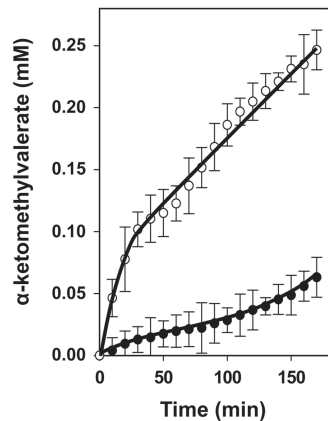


FIG 2  $\alpha$ -Ketoglutarate-driven production of  $\alpha$ -ketomethylvalerate, the  $\alpha$ -keto acid of Ile, in the absence (●) and the presence (○) of 0.2 mM L-lactate. Cells were incubated in the presence of 2 mM  $\alpha$ -ketoglutarate, 2 mM Ile, and 50  $\mu$ M PLP.

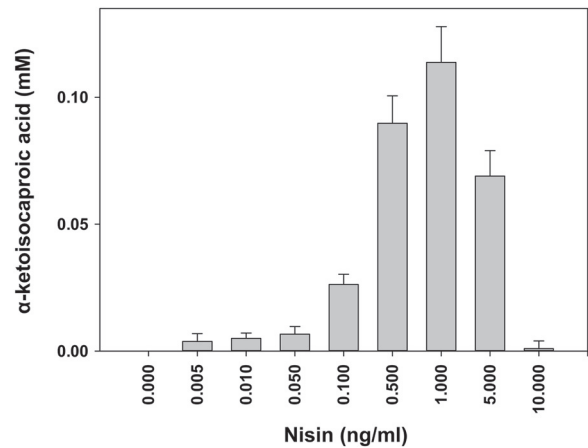


FIG 3 Production of  $\alpha$ -ketoisocaproate, the  $\alpha$ -keto acid of Leu, by resting cells of *L. lactis* IL-9000(pNZ-CitP). Cells were grown until the OD<sub>660</sub> was 0.6 in the presence of nisin added to the growth medium from the beginning at concentrations ranging from 0 to 10 ng  $\cdot$  ml<sup>-1</sup>. Resting cells were incubated for 3 h in the presence of 2 mM  $\alpha$ -ketoglutarate, 2 mM Leu, and 50  $\mu$ M PLP.

nisin. The highest production of the  $\alpha$ -keto acid  $\alpha$ -ketoisocaproic acid (115  $\mu$ M) was observed at 1 ng  $\cdot$  ml<sup>-1</sup> of nisin when resting cells were incubated with 2 mM  $\alpha$ -ketoglutarate and Leu (Fig. 3). Cells grown in the absence of the inducer did not produce any  $\alpha$ -ketoisocaproic acid. Similarly, no production was observed at higher inducer concentrations. Under these conditions cells grew significantly slower and eventually stopped growing, possibly caused by the overproduction of inactive CitP. It follows that the transamination activity of the cells follows the expression level of CitP. Unfortunately, the amount of  $\alpha$ -ketoisocaproic acid produced by resting cells of IL1403(pFL3) and IL-9000(pNZ-CitP) growing in the presence of 1 ng  $\cdot$  ml<sup>-1</sup> nisin was similar (Fig. 1D and 3), suggesting similar levels of expression with the two expression systems.

**Effect of glucose on further metabolism of  $\alpha$ -keto acids.** Enhancement of the citrate (and oxaloacetate) consumption rate by L-lactate was also observed when L-lactate was produced in the cytoplasm by glycolysis in the presence of glucose (9, 10). Surprisingly, resting cells of IL1403(pFL3) produced much smaller amounts of  $\alpha$ -keto acids after 3 h when 1 mM glucose was included with  $\alpha$ -ketoglutarate and the amino acids (Fig. 4A and B). At the same time, the amount of Glu produced by the cells was significantly higher than that observed with the cells incubated in the absence of glucose (Fig. 4C and D), suggesting high transamination activity. In the absence of glucose, the amounts of the  $\alpha$ -keto acids and Glu produced were in good agreement for all tested amino acids (Fig. 4A and C). The data suggest that in the presence of glucose, the  $\alpha$ -keto acids were not the end products. Reduction of  $\alpha$ -keto acids to  $\alpha$ -hydroxy acids by resting cells of *L. lactis* was previously observed after addition of glucose (14) and during citrate-driven transamination in an oxaloacetate decarboxylase mutant (13). Accordingly,  $\alpha$ -hydroxy acids formed from Met and Phe, i.e., 2-hydroxy-4-methylthiobutyrate and phenyllactate, respectively, were measured in the presence of glucose and not in its absence (Fig. 4E and F).  $\alpha$ -Hydroxy acids derived from branched-chain amino acids could not be measured due to limitations of the HPLC detection method, but the high production of Glu strongly suggests further metabolism of the corresponding

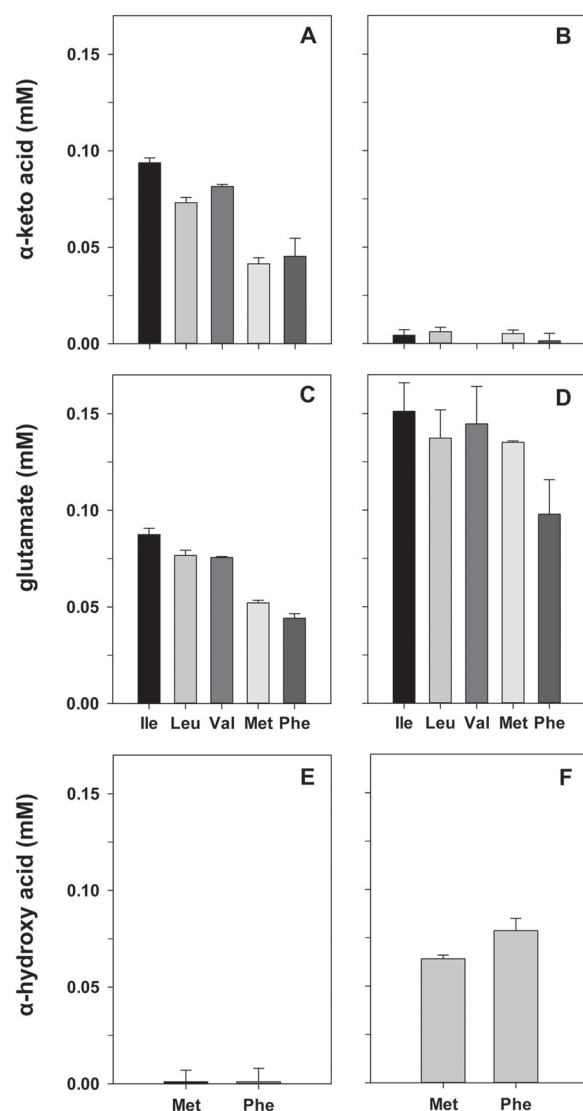


FIG 4  $\alpha$ -Ketoglutarate-driven transamination in resting cells of *L. lactis* IL1403(pFL3) in the absence (A, C, E) and presence (B, D, F) of 1 mM glucose. Concentrations of the corresponding  $\alpha$ -keto acids (A, B), glutamate in the presence of Ile, Leu, Val, Phe, and Met (C, D), and the  $\alpha$ -hydroxy acids of Met and Phe (E, F) were determined after 3 h of incubation with 2 mM  $\alpha$ -ketoglutarate, 2 mM the amino acids, and 50  $\mu$ M PLP.

$\alpha$ -keto acids (Fig. 4D). The amounts of 2-hydroxy-4-methylthiobutyrate and phenyllactate produced were smaller than the amounts of Glu produced, indicating additional conversion of the  $\alpha$ -keto acids into other flavor compounds (Fig. 4D and F). No conversion of the  $\alpha$ -keto acids produced by permeabilized cells was observed when glucose or NADH was added to the incubation mixture (not shown).

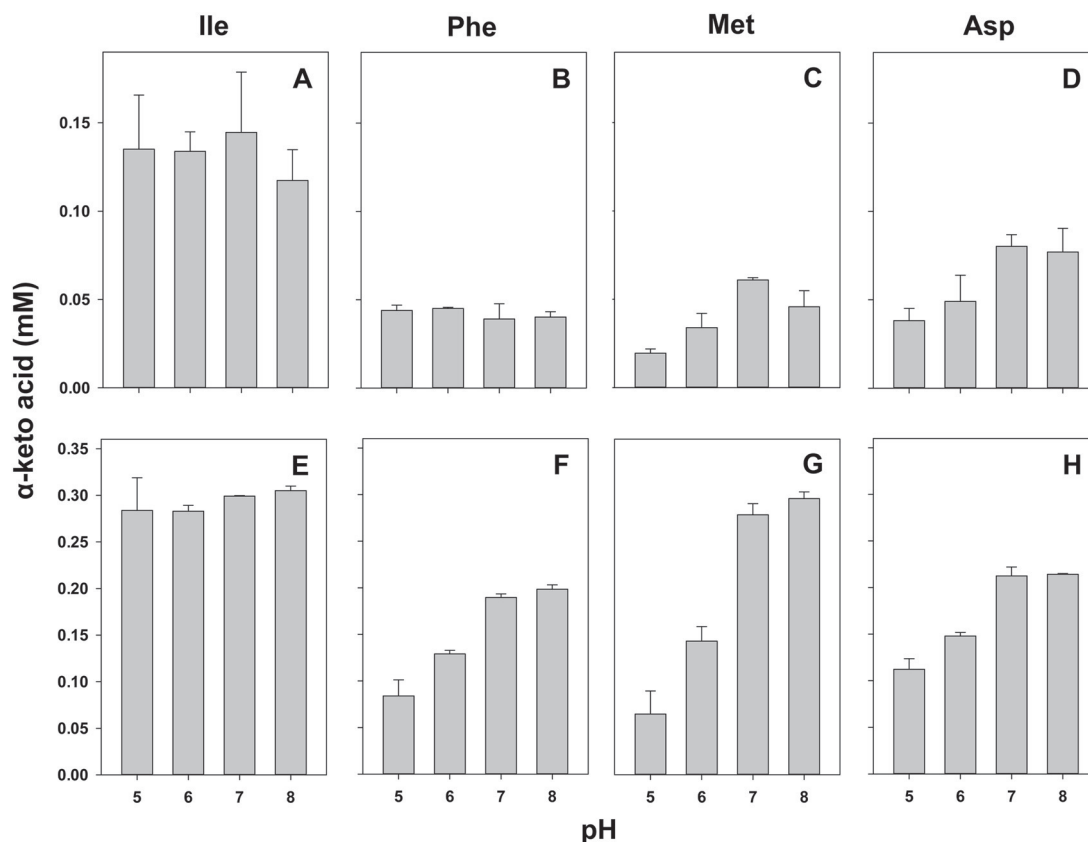
#### pH dependence of $\alpha$ -ketoglutarate-driven transamination.

BcaT and AraT, the two major transaminases of *L. lactis* that show affinity toward branched-chain amino acids, aromatic amino acids, and Met, were reported to be the most active over the pH range from 7 to 8, and therefore, most studies were performed at an external pH of 8 (1, 3). This is surprising, since the cytoplasmic pH of *L. lactis* IL1403(pFL3) under conditions where no energy source such as glucose is present is close to 6 (9, 10). The pH

profile of the aspartate transaminase AspC has not been studied (6). Here, Ile was chosen as a representative for BcaT, Phe was chosen as a representative for AraT, Met was chosen as a representative for BcaT and AraT, and Asp was chosen as a representative for AspC. The  $\alpha$ -ketoglutarate-driven transamination activity by resting cells of *L. lactis* IL1403(pFL3) was determined over the pH range from 5 to 8 by measurement of the formation of the corresponding  $\alpha$ -keto acid (Fig. 5). The process as a whole, including the transport steps, was remarkably insensitive to pH (Fig. 5A to D). A slightly lower activity was observed at the acidic side of the spectrum only with Met and Asp. Bypassing of the transport steps by treating the cells with Triton X-100 revealed the same flat profile for Ile which is representative of BcaT activity (Fig. 5E). Conversion of Phe and Asp, catalyzed by AraT and AspC, respectively, is slightly raised at higher pH (Fig. 5F and H). In line with previous studies (1), formation of the  $\alpha$ -keto acid of Met was improved at high pH (Fig. 5G). The final amounts of each  $\alpha$ -keto acid formed after 24 h were equal over the whole pH range in whole and permeabilized cells (not shown). The production of  $\alpha$ -hydroxy acids from Met and Phe at high pH in the presence of glucose was completely inhibited (not shown).

#### DISCUSSION

$\alpha$ -Ketoglutarate is the preferred keto donor in the reaction catalyzed by lactococcal transaminases that catalyze the first step in flavor development from excess amino acids in dairy fermentations. Often, the availability of cytoplasmic  $\alpha$ -ketoglutarate limits the production of flavor compounds, a limitation that is only partly overcome by adding the keto donor to the external medium (21–23). The present study clearly shows that the lack of an uptake system in the cytoplasmic membrane of strain IL1403 is the reason for this failure. While resting cells did not catalyze transamination in the presence of  $\alpha$ -ketoglutarate and a number of amino acids, significant activity was observed after disruption of the membrane, showing that the limitation was in a transport step. Since a previous study showed no limitation in import/export of amino acids (13), the limitation was clearly in the uptake of  $\alpha$ -ketoglutarate. Surprisingly, it was observed that the citrate transporter CitP found in specific strains of *L. lactis* is capable of transporting  $\alpha$ -ketoglutarate into the cell. This was supported by three experimental results: (i) significant transamination activity in strain IL1403 containing CitP (Fig. 1C), (ii) the correlation between the transamination activity of strain IL1403 and the levels of CitP expression (Fig. 3), and (iii) enhancement of the rate of transamination in the presence of L-lactate (Fig. 1D and 4). The last observation is diagnostic for substrates of CitP and relates to the very efficient citrate/L-lactate exchange mode of transport catalyzed by CitP during citrate fermentation. It follows that *L. lactis* subsp. *lactis* biovar diacetylactis, a species that ferments citrate, should specifically be used for flavor enhancement by externally added  $\alpha$ -ketoglutarate. At least in resting cells, conversion of  $\alpha$ -ketoglutarate in the transamination reactions appears to be the only pathway which ensures a high efficiency of conversion into flavor compounds. The requirement for a high pH for optimal transamination activity reported before (1, 3) was not substantiated in the present study. In fact, at the relatively acidic pH values found in food fermentations, the overall activity by the cells was significant with all amino acids tested (Fig. 5). Remarkably, in the presence of only  $\alpha$ -ketoglutarate and the amino acids, the corresponding  $\alpha$ -keto acids were the only products formed in addition to gluta-

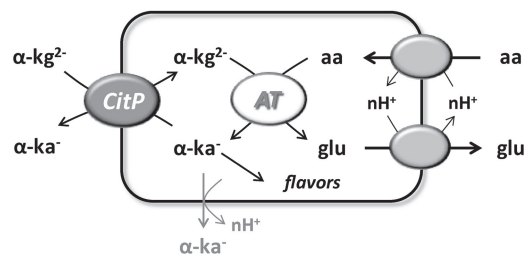


**FIG 5**  $\alpha$ -Ketoglutarate-driven transamination in resting (A to D) and permeabilized (E to H) cells of *L. lactis* IL1403(pFL3) with Ile (A, E), Phe (B, F), Met (C, G), and Asp (D, H). Concentrations of the corresponding  $\alpha$ -keto acids were determined after 3 h of incubation in 50 mM potassium phosphate buffer at the indicated pH containing 2 mM  $\alpha$ -ketoglutarate, 2 mM the amino acid, and 50  $\mu$ M PLP.

mate (Table 1), and the possibility that further conversion into flavor compounds may be even more effective in the lower, more physiological pH range cannot be excluded. Further metabolism required the presence of glucose (Fig. 4). While this is apparent for the reduction to the  $\alpha$ -hydroxy acids for which glycolysis provides the reducing equivalents, other conversions apparently rely on specific cytoplasmic factors or conditions as well.

In citrate-fermenting *L. lactis* subsp. *lactis* biovar diacetylactis, the gene encoding the citrate transporter CitP is located on an endogenous plasmid, while the genes for the metabolic enzymes are clustered in a single operon on the chromosome. Moreover, expression of the transporter gene is controlled by the pH of the growth medium rather than the presence of citrate in the medium. Both arguments suggest a wider physiological function for CitP in *Lactococcus* species than the uptake of citrate in the citrate metabolic pathway alone. To contrast the situation in *Lactococcus* spp., the expression of the *citP* gene in *Leuconostoc mesenteroides* is induced by citrate and *citP* is part of the same operon that contains the metabolic enzymes (12). For sure, uptake of citrate in exchange with the metabolic end product L-lactate during citrate/carbohydrate cometabolism is also the most prominent function of CitP in lactococci. In the absence of a carbohydrate (and L-lactate), CitP catalyzes the uptake of citrate in exchange with intermediates and/or end products of the citrate metabolic pathway, such as pyruvate,  $\alpha$ -acetylactate, and acetate (9). CitP functions in oxaloacetate metabolism by taking up oxaloacetate from the me-

dium, after which it is degraded to acetate (10). Under conditions where oxaloacetate decarboxylase activity is compromised, internalized oxaloacetate was shown to function as a  $\alpha$ -keto donor in transamination reactions, resulting in flavor compound production (13). The present study demonstrates the involvement of CitP in flavor-producing pathways driven by  $\alpha$ -ketoglutarate (Fig. 6). The pathway consists of, besides CitP, amino acid transporters and transaminases. Overall, external  $\alpha$ -ketoglutarate and an external amino acid are converted to Glu and the  $\alpha$ -keto acid derived from the amino acid. Alternatively, the amino acid may be produced inside by proteolytic activity. The produced  $\alpha$ -keto acid may be excreted by CitP in exchange with  $\alpha$ -ketoglutarate, leave the cell by passive diffusion, or be further metabolized.



**FIG 6** Schematic representation of  $\alpha$ -ketoglutarate-driven transamination in *L. lactis* IL1403(pFL3).  $\alpha$ -kg,  $\alpha$ -ketoglutarate;  $\alpha$ -ka,  $\alpha$ -keto acid; aa, amino acid; AT, aminotransferase; CitP, citrate permease; glu, glutamate.

The different physiological functions require CitP to have broad substrate specificity. Recently, CitP was characterized as a promiscuous carboxylate transporter (15). CitP translocates many mono-, di-, and tricarboxylates of the form  $X\text{-CR}_2\text{-COO}^-$ , in which X is either OH, O, or H. The set of substrates includes the  $\alpha$ -keto acids and  $\alpha$ -hydroxy acids that are formed by transamination of amino acids. It was shown that CitP catalyzes efficient transport of  $C_4$  to  $C_6$  dicarboxylates, such as succinate, glutarate, and adipate, and 2-hydroxy (L-malate) and 2-keto (oxaloacetate) forms of  $C_4$  dicarboxylates (15). The present study adds  $\alpha$ -ketoglutarate, the  $\alpha$ -keto form of the  $C_5$  dicarboxylate glutarate, to the set of substrates of CitP.

Metabolism of  $\alpha$ -ketoglutarate and amino acids in *L. lactis* IL1403(pFL3) is coupled at the level of aminotransferases (Fig. 6). Two major lactococcal transaminases, AraT and BcaT, were purified and characterized. Both are encoded on the genome of *L. lactis* subsp. *lactis* IL1403 (5) used in this study and highly similar in sequence to the characterized enzymes. Purified AraT (1, 24) is a transaminase active on aromatic amino acids (phenylalanine, tryptophan, tyrosine), leucine, and methionine, utilizing  $\alpha$ -ketoglutarate as the keto donor, activities that were also observed in the present study. The purified enzyme was most active at pH 7 to 8, while the cumulative transamination activities for these substrates in the cytoplasm of strain IL1403 were still high at the lower pH of 5.8, a condition more compatible with cheese production (4). Purified BcaT (2) is active with the branched-chain amino acids and methionine, utilizing  $\alpha$ -ketoglutarate as a keto donor (2), again, activities observed in the cytoplasm of IL1403. The highest activity of the purified enzyme was observed at pH 7.5, while transamination of the branched-chain amino acid isoleucine driven by  $\alpha$ -ketoglutarate in IL1403(pFL3) was not sensitive to pH over the pH range from 5 and 8. A third transaminase annotated in the genome of strain IL1403 (5, 6), the aspartate transaminase AspC, was purified from *Brevibacterium linens* 47 and shown to be active on aspartate and  $\alpha$ -ketoglutarate with an optimal pH of 8.5 to 9 (25). Little effect of pH on the same activity was shown in the cytoplasm of *L. lactis* IL1403. The amino acid substrate profile of  $\alpha$ -ketoglutarate transamination activity in the cytoplasm of IL1403 reported here is very similar to that of the recently reported oxaloacetate transamination activity in the same strain (13), while purified AraT and BcaT did not show activity with the latter keto donor. The discrepancies between the activities observed in the cytoplasm of strain IL1403 and the properties of the purified transaminases suggest that purification alters the properties of the enzymes or that other unknown transaminases are active in IL1403.

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